NOVEL PHYTOCHROME-INTERACTING PROTEIN AND A USE THEREOF

Technical Field

The present invention relates to a novel protein involved in phytochrome light signal transduction mechanisms and a use thereof, and more particularly, to a type 5 serine/threonine phosphatase interacting with phytochromes and a use thereof.

Background Art

To perceive the status of their light environment, plants have evolved various photoreceptors. The photoreceptors (an unidentified UV-B receptor, a phototropin and cryptochrome sensing UV-A/blue light region of the spectrum, and phytochromes sensing red(R)/far-red(FR) region of the spectrum) mediate signals to genes regulating the growth and development of plants (Fankhauser, C. & Chory, J. *Curr. Biol.*, 9:R123-R126, 1999; Neff, M. M., *et al.*, *Genes Dev.*, 14:257-271, 2000). The recent development of molecular biological and biochemical research technologies and molecular genetic research technologies have been made in the molecular cloning and genetic characterization of the photoreceptors themselves, as well as some signal intermediate components involved in transducing perceived signals from photoreceptors to photoresponsive genes (Quail, P. H. *Curr. Opin. Cell. Biol,*. 14:180-188, 2002; Gyula, P. *et al.*, *Curr. Opin. Plant Biol.*, 6:446-452, 2003).

Phytochromes are photoreceptors whose characteristics were most well studied, that regulate various aspects of the growth and development of higher plants. Depending to the spectrum of light irradiated on phytochromes, a reversible photo-conversion occurs between a biologically inactive, red light-absorbing form (Pr) and a

biologically active, far-red light-absorbing form (Pfr). The photo-conversion into the Pfr form by red light treatment initiates translocation from the cytoplasm of phytochromes themselves to the nucleus, and activates the signal transduction pathway inducing various effects on the expression and development of genes, thus regulating the growth and development of plants (Quail, P. H. *Curr. Opin. Cell. Biol,*. 14:180-188, 2002; Fankhauser, C. & Chory, J. *Curr. Biol.*, 9:R123-R126, 1999). It is reported that there are five different phytochromes (designated phyA, phyB, phyC, phyD and phyE) in *Arabidopsis thaliana* (Neff, M. M., *et al.*, *Genes Dev.*, 14:257-271, 2000; Quail, P. H. *Curr. Opin. Cell. Biol,*. 14:180-188, 2002).

The generic phytochromes consist of an apoprotein of about 116-127 kDa and a tetrapyrrole chromophore, phytochromobillin that is covanlently linked to the apoprotein (Quail, P. H. *Curr. Opin. Cell. Biol,*. 14:180-188, 2002; Gyula, P. *et al.*, *Curr. Opin. Plant Biol.*, 6:446-452, 2003). The photosensoty activity of phytochromes resides in their unique capacity for reversible, light-induced interconversion between the Pr form and the Pfr form. The monomer of phytochrome molecule is composed of a globular N-terminal domain (~ 70 kDa), which is anchoring the chromophore, and a C-terminal domain linked via a flexible hinge region. The N-terminal domain is responsible for photosensory function. Also, the conformationally open C-terminal domain (about 55 kDa) is known to be involved in signal transfer (Quail, P. H. *Curr. Opin. Cell. Biol,*. 14:180-188, 2002; Gyula, P. *et al.*, *Curr. Opin. Plant Biol.*, 6:446-452, 2003). The C-terminal domain contains a pair of the Per-Arnt-Sim (PAS) motifs around the regulatory core region. The PAS motifs are known to be involved in protein-protein interaction and inter-domain communications in some sensory proteins. The results of analysis with recombinant oat phytochrome A showed that the C-terminal domain of phytochromes

possesses serine/threonine protein kinase activity (Yeh, K. C. & Lagarias, J. C. *Proc. Natl. Acad. Sci. U.S.A.* 95:13976-13981, 1998; Fankhauser, C. *et al.*, *Science,* 284:1539-1541, 1999). Furthermore, it was suggested by the results of spectral and photochemical tests that the photo-isomerization of phytochromes induced by chromopores triggers conformational changes throughout the whole phytochrome molecule via inter-domain communication within the molecules, like the well-characterized rhodopsin visual receptor in animals (Maeda, T. *et al.*, *Prog. Retin. Eye Res.*, 22:417-434, 2003; Vishnivetskiy, S. A. *et al.*, *J. Biol. Chem.*, 275:41049-41057, 2000). In addition, the conformational signals of phytochrome could be further differentiated by inter-domain interactions in the phytochrome molecule, and this is presumed to be modulated by reversible phosphorylation/dephosphorylation at serine residue in the hinge region. In spite of these many authentic findings, however, minute mechanisms by which the phytochromes transduce light signals to photoresponsive genes are not yet completely established.

Disclosure of the Invention

Therefore, during extensive studies to establish the light signal transduction mechanism of phytochromes and to identify new mediator molecules involved therein, the present inventors have found a novel protein interacting with phytochromes and identified the functions and characteristics thereof, thus completing the present invention. Accordingly, an object of the present invention is to provide a novel protein interacting with phytochromes and use thereof.

To achieve the above object, in one aspect, the present invention provides an isolated polypeptide having an amino acid sequence set forth in SEQ ID NO: 4 or an

amino acid sequence having a homology of at least 70% with the amino acid sequence of SEQ ID NO: 4.

In another aspect, the present invention provides an isolated polynucleotide having a nucleotide sequence encoding said polypeptide or a nucleotide sequence complementary to said nucleotide sequence, and a recombinant vector comprising the same.

Also, the present invention provides a cell comprising said recombinant vector.

In still another aspect, the present invention provides a method for producing a plant sensitive to light signal transduction, comprising introducing said polynucleotide encoding the polypeptide into a plant.

In still another aspect, the present invention provides a method for producing a dwarf plant, comprising introducing a polynucleotide encoding the sequence of amino acids 1-138 of SEQ ID NO: 4 into a plant.

In yet another aspect, the present invention provides a method of identifying a phytochrome signal transduction-associated substance using said polypeptide or a polynucleotide encoding the polypeptide.

Also, the present invention provides a method of identifying a plant dwarfism-causing substance using a polypeptide encoding the sequence of amino acids 1-138 of SEQ ID NO: 4 or a polynucleotide encoding the polypeptide.

Furthermore, the present invention provides a method of preparing a protein having phosphatase activity using said polynucleotide encoding the polypeptide.

Hereinafter, the present invention will be described in detail.

The present invention provides novel protein PAPP5 interacting with phytochromes. The PAPP5 protein is a kind of type 5 serine/threonine protein phosphatase, and its N-terminal region has three TPR (tetratricopeptide repeats) motifs which are involved in interaction with phytochromes. The C-terminal region of the inventive PAPP5 protein exhibits phosphatase activity. This enzymatic activity is inhibited by an okadaic acid, and on the contrary, is promoted by an arachidonic acid. This enzymatic activity of the PAPP5 protein is regulated by an allosteric change caused by a TPR domain at N-terminus. Also, the PAPP5 has the activity of dephosphorylating autophosphorylated phytochromes, mainly the Pfr phytochrome.

The polypeptide according to the present invention includes a polypeptide having an amino acid sequence of SEQ ID NO: 4 and functional equivalents thereof. As used herein, the term "functional equivalents" refers to polypeptides having substantially the same physiological activity as the protein of SEQ ID NO: 4, which have a sequence homology of at least 70%, preferably at least 80%, and more preferably at least 90% with the amino acid sequence of SEQ ID NO: 4, as a result of the addition, substitution or deletion of amino acids. As used herein, "substantially the same physiological activity" means phosphatase activity. The functional equivalents include, for example, amino acid sequence variants with substitutions, deletions or substitutions in some of the amino acids of the polypeptide having the amino acid sequence of SEQ ID NO: 4. Preferably, the substitutions of amino acid is conservative substitutions. Examples of conservative substitutions of amino acid occurring in nature are as following: Aliphatic amino acids (Gly, Ala, Pro), hydrophobic amino acids (Ile, Leu, Val), aromatic amino acids (Phe, Tyr, Trp), acidic

amino acid (Asp, Glu), basic amino acids (His, Lys, Arg, Gln, Asn) and sulfur-containing amino acids (Cys, Met). The deletions of amino acids are preferably located in portions which are not involved directly in the physiological activity of PAPP5. A preferred functional equivalent of the inventive polypeptide may be a polypeptide (SEQ ID NO: 14) with deletions of amino acids 1-138 in SEQ ID NO: 4. The polypeptide with the deletions has a homology of 77.8% to the PAPP5 protein. Furthermore, the scope of the functional equivalents also encompasses polypeptide derivatives having partial modifications of the chemical structure of the inventive polypeptide while maintaining the basic backbone and physiological activity of the inventive polypeptide. For example, it encompasses structural modifications for modifying the stability, storage, volatility or solubility of the inventive polypeptide.

The inventive polypeptide may be extracted from the nature (e.g., plant cells) or obtained by the expression of a recombinant nucleic acid encoding the inventive polypeptide or by chemical synthesis. Preferably, it can be isolated from *Arabidopsis thaliana*. The inventive polypeptide may be easily prepared by any chemical synthesis method known in the art (Creighton, Proteins; Structures and Molecular Principles, W. H. Freeman and Co., NY, 1983). Typical synthesis methods include, but are not limited to, liquid or solid phase synthesis, fragment condensation, F-MOC or T-BOC chemical method (Chemical Approaches to the Synthesis of Peptides and Proteins, Williams *et al.*, Eds., CRC Press, Boca Raton Florida, 1997; A Practical Approach, Athert on & Sheppard, Eds., IRL Press, Oxford, England, 1989).

Furthermore, the inventive polypeptide may also be constructed by a genetic engineering method. For this purpose, a DNA sequence encoding a PAPP5 or

fragment thereof is first constructed according to the conventional method. The DNA sequence may be constructed by performing PCR amplification with suitable primers. Moreover, the DNA sequence may also be synthesized by the standard methods known in the art, for example, using an automatic DNA synthesizer (Biosearch or Applied Biosystem). The constructed DNA sequence is operably linked to expression control sequences and inserted into a vector containing one or more expression control sequences (e.g., promoters, enhancers, etc) that control the expression of the DNA sequence. Host cells are transformed with the resulting vector. The transformed cells are cultured in suitable medium and conditions for the expression of the DNA sequence, and a substantially pure polypeptide encoded by the DNA sequence is collected from the cell culture. The collection can be performed by any method known in the art (e.g., chromatography). As used herein, "substantially pure polypeptide" means that the polypeptide according to the present invention substantially contains no other proteins derived from host cells. For further information on the genetic engineering method for the synthesis of the inventive polypeptide, see the following references: Maniatis *et al.*, Molecular Cloning; A laboratory Manual, Cold Spring Harbor laboratory, 1982; Sambrook *et al.*, supra; Gene Expression Technology, Method in Enzymology, Genetics and Molecular Biology, Method in Enzymology, Guthrie & Fink (eds.), Academic Press, San Diego, Calif, 1991; and Hitzeman *et al.*, J. Biol. Chem., 255:12073-12080, 1990.

In another aspect, the present invention provides an isolated polynucleotide having a nucleotide sequence encoding the PAPP5 and functional equivalents thereof. The polynucleotides include DNA, cDNA and RNA sequences. Namely, the polynucleotide may have a nucleotide sequence encoding either an amino acid

sequence of SEQ ID NO: 4 or an amino acid sequence having a homology of at least 70% with the amino acid sequence of SEQ ID NO: 4, or a nucleotide sequence complementary to said nucleotide sequence. The polynucleotide may preferably have a nucleotide sequence set forth in SEQ ID NO: 3 or SEQ ID NO: 15.

The inventive polynucleotide may be operably linked to expression control sequences. The term "operably linked" means that one nucleic acid fragment binds to other nucleic acid fragment so that the function or expression of one is affected by the other. Also, the term "expression control sequence" refers to a DNA sequence which controls the expression of operably linked nucleic acid sequences in certain host cells. Such expression control sequence includes a promoter for initiation of transcription, an optional operator sequence for control of transcription, a sequence encoding suitable mRNA ribosomal binding sites, and sequences which control the termination of transcription and translation.

The inventive polynucleotide may be inserted into a suitable expression vector. As used herein, the term "expression vector" refers to a plasmid, virus or other mediator known in the art, into which the inventive polynucleotide can be inserted. Vectors suitable for introducing the inventive polynucleotide into plant cells include, but are not limited to, Ti-plasmids, root-inducing (Ri)-plasmids and plant virus vectors. Preferably, a pNB96 vector may be used.

The recombinant vector comprising the inventive polynucleotide may be introduced into a cell using any method known in the art. The cell may be a eukaryotic cell, such as yeast and plant cell, or a prokaryotic cell, such as *E. coli*. Preferably, the cell may be *E. coli* cell or *Agrobacterium* sp. cells The known method which can be used to introduce the inventive vector into host cell includes, but are not limited to, *Agrobacterium*-mediated transformation, particle gun

bombardment, silicon carbide whiskers, sonication, electroporation, and PEG (polyethylenglycol) precipitation. In still another aspect, the present invention provides the cell transformed with the inventive recombinant vector. The cell includes, but is not limited to, a eukaryotic cell, such as yeast and plant cell, or a prokaryotic cell, such as *E. coli*.

In still another aspect, the present invention provides a method for producing a plant sensitive to light signal transduction by overexpressing the polynucleotide encoding the polypeptide in the plant. This method comprises the steps of:

(a) inserting a polynucleotide encoding the inventive PAPP5 or a functional equivalent thereof into an expression vector; and

(b) introducing the expression vector into a plant.

The polynucleotide may have a nucleotide sequence set forth in SEQ ID NO: 3 or SEQ ID NO: 15. The expression vector which can be used in the above method may be preferably a vector comprising a promoter inducing the overexpresion of gene (e.g., CaMV 35S promoter). For example, there is a pNB96 vector. As used herein, the term "overexpression" means that a gene is expressed at a higher level than that in wild-type plants. The method which can be used to introduce the inventive polynucleotide-containing expression vector into the plant is as described above. Preferably, the *Agrobacterium*-mediated transformation may be used.

In yet another aspect, the present invention provides a transgenic plant produced by the above method. The inventive polypeptide-overexpressing plant produced by the above method has a characteristic in that it is sensitive to light signal transduction. Namely, it shows strong de-etiolation (a short hypocotyl phenotype

that is the phenomenon of light sensitivity) in a continuous red light-high irradiance response (Rc-HIR) and a continuous far-red light-high irradiance response (FRc-HIR), as compared to wild-type plant. Also, it strongly shows an "End-Of-Day Far-Red (EOD-FR) response that is mediated by a phytochrome B, and anthocyanin accumulation that is a phyrochrome A-mediated response, as compared to wild-type plants. Since the PAPP5-overexpressing transgenic plant is sensitive to light, it has an advantage in that its growth is not inhibited even under the condition of weak intensity light or small amount light so that the plant can be normally grown. In yet another aspect, the present invention provides a plant tissue or seed derived from the transgenic plant.

In still another aspect, the present invention provides a method for producing a dwarf plant by overexpressing a polynucleotide encoding a fragment of said polypeptide in a plant. This method comprises the steps of:

(a) introducing a polynucleotide encoding the sequence of amino acids 1-138 of SEQ ID NO: 4 into an expression vector; and

(b) introducing the expression vector into a plant.

The polynucleotide encodes the TPR domain of the inventive polypeptide PAPP5. Preferably, the polynucleotide has a nucleotide sequence encoding the sequence of amino acids 1-138 of SEQ ID NO: 4. The expression vector into which the polynucleotide is introduced, and the method for introducing the expression vector into the plant, are as described above for the production method of the light-sensitive plant. Preferably, the pNB96 vector and the *Agrobacterium*-mediated transformation may be used. Furthermore, the present invention provides a transgenic plant produced by said method and a plant tissue and seed derived the transgenic plant.

The TPR domain-overexpressing transgenic plant produced by said method shows dwarf phenotypes, such as shorter height multiple shoots, floral shoot internodes, as compared to wild-type plant.

The plant to which the inventive methods can be applied may be a dicotyledonous plant or a monocotyledonous plant. The dicotyledonous plant includes soy bean, *Arabidopsis thaliana,* tobacco plant, eggplant, red pepper, petunia, potato, tomato, Chinese cabbage, rape, cabbage, cotton plant, lettuce, peach, pear, strawberry, watermelon, melon, cucumber, carrot and celery. The monocotyledonous plant includes rice, barley, wheat, rye, corn, sugar cane, oat, and onion.

The PAPP5 according to the present invention is a protein involved in phytochrome signal transduction, which interacts with phytochrome A and phytochrome B. The TPR domain located at the N-terminus of the PAPP5 protein is involved in the interaction between PAPP5 and phytochromes, and the C-terminal domain of the PAPP5 has phosphatase activity. The PAPP5 is activated by autophosphorylated Pfr phytochrome and has the enzymatic activity of dephosphorylating the autophosphorylated phytochrome. Thus, the present invention provides a method of identifying a phytochrome signal transduction-associated substance using the PAPP5 protein, functional equivalents thereof, or polynucleotides encoding the same. The phytochrome signal transduction-associated substance identified by this method may be one having the activity of increasing or inhibiting the activity, expression and/or intracellular level of the inventive polypeptide or a polynucleotide encoding the polypeptide. The substance may be one having the

same or similar activity as the inventive polypeptide or polynucleotide. Alternately, the substance may also be one involved in phytochrome signal transduction by interaction with the inventive polypeptide or the polynucleotide encoding the polypeptide. The substance includes, but is not limited to, polynucleotide, polypeptide, chemical or natural extract.

The above method can be performed using the inventive polypeptide or the polynucleotide encoding the same, as a probe. As used herein, the term "probe" refers to a mediator for identifying the desired substance. For example, the above method may be performed by analyzing the binding pattern between a candidate substance and the inventive polypeptide or polynucleotide using the polypeptide or the polynucleotide as a probe. Alternatively, this method may be performed by contacting the inventive PAPP5 with a candidate substance to identify a substance that inhibits or increases the activity of the PAPP5. In this case, the method may comprise the steps of culturing a candidate substance along with recombinant cell expressing the inventive PAPP5, and measuring the effect of the candidate substance on an increase in the activity or intracellular level of the PAPP5.

Alternatively, this method may also be performed by identifying a gene having the same or similar function as the inventive gene from other plants, through hybridization between the inventive polynucleotide or fragment thereof and cDNA prepared from RNA or mRNA extracted from other plants. The method may also be performed by identifying either a substance that binds directly to the polynucleotide or a substance that inhibits or activate the expression of the polynucleotide. In addition, this method may comprise performing a sequence homology search program known in the art using the inventive PAPP5 or polynucleotide encoding the same, so as to

identify a protein or gene having high homology with the PAPP5 or polynucleotide encoding the same.

The above identification may be performed by various methods generally used in the art, including, but not limited to, cDNA library screening, BAC (bacterial artificial chromosome) screening, DNA chip, protein chip, polymerase chain reaction (PCR), Northern blot, Southern blot, Western blot, enzyme-linked immunosorbent assay (ELISA), 2-D gel analysis, yeast 2-hybrid system, and *in vitro* binding assay.

The inventive polypeptide or the polynucleotide encoding the polypeptide may be labeled with radioactive isotope, fluorescent dye or light development enzyme, in order to facilitate the screening and isolation of a substance to be identified. Preferably, it may be labeled with ^{3}H, ^{32}P, ^{35}S, FITC (fluorescein isothiocyanate), TRITC (tetramethylrhodamine isothiocyanate), biotin, digoxigennin, HRP (horse-radish peroxidase), glucose oxidase, alkaline phosphatase, or the like. Labeling methods are known in the art. For example, a nucleic acid may be labeled by a method of uniformly labeling the entire nucleic acid using, for example, nick translation, random oligonucleotide primers, or a method of labeling the 5'- or 3'-terminal region, such as kination or filling-in. Also, a polypeptide may be labeled by radioactive oxonation. The tyrosine or histidine of the polypeptides can be labeled directly with radioiodine. It also may be labeled with Chloramine-T, Iodogen or lactoperoxidase.

Furthermore, the TPR domain of the inventive PAPP5 protein (the sequence of amino acids 1-138 of SEQ ID NO: 4) is involved in the interaction between the PAPP5 and phytochromes, as described above, and if it is oversxpressed in plants, it will cause plant dwarfism. It was first found in the present invention that the

overexpression of the TPR domain causes dwarfism in plants. Thus, the present invention provides a method of identifying a plant dwarfism-causing substance using the TPR domain or a polynucleotide encoding the same, as a probe. This method allows the identification of a substance that indirectly or directly causes dwarfism in plants either by inducing or promoting the expression of the TPR domain of the inventive PAPP5 protein or by interacting with the TPR domain. The identification may be performed by the above-described methods.

In another aspect, the present invention provides a method of producing a protein having phosphatase activity using the inventive polypetide or a polynucleotide encoding the polypeptide. This method comprises the step of:

(a) introducing a polynucleotide encoding a polypeptide having either an amino acid sequence of SEQ ID NO: 4 or an amino acid sequence having a homology of at least 70% with said amino acid sequence into an expression vector;

(b) introducing the expression vector into a cell;

(c) culturing the cell to express the polynucleotide; and

(d) collecting the cultured protein from the cell culture .

The polynucleotide which can be used in this method includes a polynucleotide encoding an amino acid sequence set forth in SEQ ID NO: 4, and a polynucleotide encoding a polypeptide having an amino acid sequence having a homology of at least 70% with said amino acid sequence. Preferably, a polynucleotide that encodes either a polypeptide of SEQ ID NO: 4 or a polypeptide (SEQ ID NO: 14) with a deletion of amino acids 1-138 of SEQ ID NO: 4 may be used. More preferably, a polynucleotide having a nucleotide sequence of SEQ ID

NO: 3 or SEQ ID NO: 15 may be used. In this method, the cell may be a eukaryotic cell, such as yeast, or a prokaryotic cell, such as *E. coli* cell.

In one embodiment of the present invention, in order to identify a new protein interacting with phytochromes, an *Arabidopsis thaliana* cDNA library was screened using a yeast 2-hybrid system. In this case, the full-length cDNA of phytochrome A was used as a bait (FIG. 1). We analyzed deduced amino acid sequences of phytochrome-interacting positive clones resulted from the yeast 2-hybrid screening. As a result, it could be seen that one clone among the clones is a kind of type 5 serine/threonine protein phosphatase (PP5). The N-terminal region of the PP5 protein has 3-4 TPRs (tetratricopeptide repeats), which are implicated in protein-protein interaction (Das, A. K., *et al.*, *EMBO J.*, 17:1192-1199, 1998; Skinner, J. *et al.*, *J. Biol. Chem.*, 272:22464-22471, 1997; Ollendorff, V. *et al.*, *J. Biol. Chem.*, 272:32011-32018, 1998; Chinkers, M. *Trends Endocrinol. Metab.*, 12:28-32, 2001), and a domain containing 3 TPRs was also present in the N-terminal region of the selected positive clone (see FIGS. 2 and 3). Also, the C-terminal region of the selected clone contains the highly conserved phosphatase domain, which contains a motif necessary for serine/threonine phosphatase activity and a sequence (-SAPNC-) whose phosphatase activity is inhibited by binding with an okadaic acid (see FIG. 3). The present inventors named the selected positive clone "*PAPP5*" (phytochrome-associated protein phosphatase 5).

The analyses of *in vivo* and *in vitro* protein-protein interactions showed that the inventive PAPP5 protein interacted specifically with phytochromes (see FIGS. 4 and 5), and in these interactions, the TPR domain at N-terminus of the PAPP5 protein was involved (see FIG. 6). Furthermore, the enzymatic activity of the PAPP5 protein

was determined using a general inorganic substrate of phosphatases, para-nitrophenol phosphate (ρ-NPP). As a result, it could be seen that the PAPP5 protein has allosteric conformational change-dependent activity. This conformational change was induced by an arachidonic acid *in vitro* (see FIG. 7), suggesting that TPR domain is an autoinhibitory region.

Moreover, it was found that the PAPP5 protein effectively dephosphorylated the autophosphorylated phytochrome *in vitro*, and the phosphatase activity was regulated by the wavelength of light (see FIG. 11). Particularly, the dephosphorylation of phytochromes by PAPP5 was strong, mainly in the Pfr phytochrome. The *in vivo* and *in vitro* results suggest that the PAPP5 is a regulator of phytochrome-mediated light signalling pathways. Also, the results suggest that the reversible phosphorylation/dephosphorylation of phytochromes in which the PAPP5 protein is involved plays an important role in the biological activity of phytochromes and the regulation of phytochrome-mediated light signal transduction.

In another embodiment, the phosphatase activity of an N-terminus or C-terminus-deleted mutant of the PAPP5 protein was examined, and as a result, it was confirmed that the N-terminus-deleted mutant of the PAPP5 maintained the phosphatase activity intact (see C of FIG. 7).

Since the phosphatase activity of PP5s is known to be promoted by an arachidonic acid (Das, A. K., *et al.*, *EMBO J.*, 17:1192-1199, 1998; Skinner, J. *et al.*, *J. Biol. Chem.*, 272:22464-22471, 1997; Ollendorff, V. *et al.*, *J. Biol. Chem.*, 272:32011-32018, 1998; Chinkers, M. *Trends Endocrinol. Metab.*, 12:28-32, 2001), whether the enzymatic activity of the PAPP5 protein is induced by the arachidonic acid was examined. As a result, it could be seen that the phosphatase activity of the

full-length PAPP5 was also induced by the arachidonic acid at high level (see FIG. C of FIG. 7). Meanwhile, the N-terminus-deleted mutant of the PAPP5 protein showed an equal or higher activity than the arachidonic acid-induced phosphatase activity of the PAPP5 protein, and the activity was independent of arachidonic acid.

In still another embodiment of the present invention, a knock-out mutant of PAPP5 and a PAPP5-overexpressing plant were produced and examined for their Rc-HIR and FRc-HIR phenotypes. As a result, the knock-out mutant of PAPP5 showed a long hypocotyl phenotype in contrast with the PAPP5-overexpressing phenotype (see FIG. 9), and also diminished rates of light-induced hook opening and cotyledon separation, reduced cotyledon expansion, and early flowering (data not shown). On the other hand, the PAPP5-overexpressing plant showed a shorter hypocotyl phenotype than that of a wild-type plant (see FIG. 9). It also strongly showed an "end-of-day far-red" (EOD-FR) response that is a phytochrome B-mediated response, and anthocyanin accumulation that is a phytochrome A-mediated response (date not shown), as compared with those of a wild-type plant. Such results indicate that the PAPP5 protein functions as a positive regulator in phytochrome A and phytochrome B signaling pathways.

In yet another embodiment of the present invention, in order to further examine the roles of the TPR domain of PAPP5, a reverse genetic approach with dominant negative mutation was used. For this purpose, the TPR domain of PAPP5 (a polypeptide consisting of the sequence of amino acids 1-138 of SEQ ID NO: 4) was overexpressed in wild-type plants. As a result, the TPR domain-overexpressed

plants showed phenotypes similar to those caused by gibberellin deficiency, and also dwarf phenotypes, including short height, multiple shoots and floral shoot internodes.

Brief Description of the Drawings

FIG. 1 is a schematic diagram of a bait for yeast 2-hybrid system, constructed to select a phytochrome A-interacting protein from the *Arabidopsis thaliana* cDNA library.

partitioned rectangle: chromophore

PSD: photosensory domain

PRD: PAS (Per-Arnt-Sim)-associated domain

HKRD: histidine kinase-associated domain

FIG. 2 schematically shows a *PAPP5* gene in the Arabidopsis thaliana genome and a PAPP5 protein encoded from the gene.

FIG. 3 shows the results of multiple alignment of the amino acid sequences of the inventive PAPP5 protein and type 5 serine/threonine protein phosphatases isolated from several species.

H. sap PP5: *Homo sapiens* PP5 (GenBank accession No. CAA61595);

M. mus PP5: *Mus musculus* PP5 (GenBank accession No. AAB70573);

R. nor PP5: *Rattus norvegicus* PP5 (GenBank accession No. CAA54454);

S. cer PP5: *Saccharomyces cerevisiae* PP5 (GenBank accession No. CAA58158);

D. meg PP5: *Drosophila melanogaster* PP5 (GenBank accession No. CAB99478); and

C. ele PP5: *Caenorhabditis elegans* PP5 (GenBank accession No. CAC51076).

FIG. 4 shows the constructions of each of a bait and prey used in yeast 2-hybrid analysis to examine the interaction between the inventive PAPP5 and phytochrome A, and the results of the yeast 2-hybrid analysis using the bait and the prey.

FIG. 5 shows the results of *in vitro* binding assay conducted to examine the interaction between a fusion protein (GST-PAPP5) of gluthathione-S-transferase (GST) and PAPP5, and phytochrome A or phytochrome B.

Fig. 6 shows the results of pull-down analysis conducted to identify a region interacting with phytochromes in the inventive PAPP5 protein (A), and the results of quantitative yeast 2-hybrid interaction assay using fragments of PAPP5.

FIG. 7 shows the results of *in vitro* phosphatase assay conducted to examine the enzymatic activity of the inventive PAPP5 protein.

A: results of analysis conducted using ρ-nitrophenol phosphate (ρNPP) as a substrate at an arachidonic acid concentration of 100 μM;

B: analysis results for catalytic effects caused by the addition of arachidonic acid at a ρ-NPP concentration of 100 mM; and

C: analysis results for enzymatic activities of PAPP5 and its domain fragments caused by the addition of arachidonic acid.

FIG. 8 is a schematic diagram showing an insertion of T-DNA in *PAPP5* gene-knock out mutants (*papp5-1* and *papp5-2*) (A), and the result showing the expression level of the PAPP5 gene in the knock-out mutants, as compared to those in wild-type plants (Col-0, Ws-2) and PAPP5-overexpressing plants (*PAPP5-OX1* and *PAPP5-OX2*) (B).

FIG. 9 shows the results of measurement for the photoresponsiveness of a knock-out mutant (*papp5-1*) and an PAPP5-overexpressing plant (*PAPP5-OX2*), as

compared to a wild-type plant (Col-0) and phythochrome mutants (*phyA-211* and *phyB-9*).

Rc-HIR: red light irradiation.

FRc-HIR: far-red-light irradiation.

FIG. 10 shows the results of analysis for the expression levels of photoresponsive genes (*CAB2, RBCS* and *CHS*) in knock-out mutants (*papp5-1* and *papp5-2*) and PAPP5-overexpressing plants (*PAPP5-OX1* and *PAPP5-OX2*) upon red light or far-red-light irradiation, as compared to those in wild-type plants (Col-0 and Ws-2).

FIG. 11 shows the results of measurement of the dephosphorylation activity of PAPP5 on autophosphorylated oat phytochrome A (phyA).

A: results of measurement of dephosphorylation activity of PAPP5 and its domain fragments;

25% glycerol and GST: control groups;

GST-TPR: a fusion protein of GST and TPR domain of PAPP5;

GST-PP2Ac: a fusion protein of GST and catalytic domain having similarity with type 2A phosphatase of PAPP5 (PP2A)

GST-PAPP5: a fusion protein of GST and full-length PAPP5

B: measurement results for dephosphorylation activity of PAPP5 according to light absorption pattern of oat phytochrome A.

FIG. 12 shows the schematic construction of a vector overexpressing the TPR domain of PAPP5 (A), and the appearance of T_1, T_2 and T_3 generations of TPR domain-overexpressing plants introduced with the vector (B).

Best Mode for Carrying Out the Invention

The present invention will be described in detail by examples. It is to be understood, however, that these examples are provided for illustrative purpose only and are not construed to limit the scope of the present invention.

<Example 1>

Yeast two-hybrid screening

The present inventors used the yeast-2 hybrid system (DupLEX-A™, OriGene Technologies) to search for proteins binding to phytochromes. First, a cDNA library was prepared from 3-week-old *Arabidopsis thaliana* according to any method known in the art. Each of cDNA fragments was inserted into a pJG4-5 plasmid (OriGene Technologies) (preparation of a prey). Meanwhile, a bait was constructed by linking phytochrome A gene *PHYA* to the LexA-DNA binding domain of pGilda (OriGene Technologies) (see FIG. 1). For this purpose, a phytochrome A gene was cloned using primers of SEQ ID NO: 1 and SEQ ID NO: 2 from a *PHYA* cDNA clone provided by Dr. Joanne Chory, Salk Institute for Biological Studies, San Diego, USA. At this time, the PCR reaction consisted of predenaturation of template DNA for 5 min at 94 °C, and then, 35 cycles of 30 sec at 94 °C, 30 sec at 50 °C and 2 min and 30 sec at 72 °C, followed by 15 min at 72 °C. Thereafter, an EGY48 yeast reporter strain (OriGene Technologies) introduced with a pSH18-34 reporter plasmid was co-transformed with the Arabidopsis thaliana cDNA fragment-containing prey and the *PHYA* gene-containing bait. A total of 7×10^6 colonies were screened, and as a result, about 150 colonies showing a positive signal to Leu- and β-galactosidase were obtained.

<Example 2>

Analysis of nucleotide sequences of positive clones

From the positive clones obtained in the yeast 2-hybride screening of Example 1, plasmids were isolated and then, the nucleotide sequence of each of the cDNA clones was determined. Next, homology searching was performed using the *Arabidopsis thaliana* genome database. As a result, it was found that one of the cDNA clones shows a homology with the sequences in BAC clones F14N22 and F7D19 on chromosome 2. The sequence analysis of the cDNA clone revealed that 13 exons and 12 introns existed in the coding region of the cDNA clone (see FIG. 2), and an open reading frame (ORF) encoding 484 amino acids was included. Also, its molecular weight was estimated to be 54 kDa. The deduced amino acid sequence was analyzed by NCBI BLAST, and as a result, a protein encoded from the cDNA clone showed high homology with type 5 serine/threonine protein phosphatases (PP5s) from other several species. The amino acid sequence of the protein was subjected to pairwise alignment with other several PP5s (see FIG. 3), and as a result, the overall identity was 50-57%, and the C-terminal catalytic domain showed a homology of 54-62% which is slightly higher than the overall identity. However, the similarity of the amino acid sequences was very high (more than 70%).

Meanwhile, the results of PROSITE analysis revealed that TPR (tetratricopeptide repeat) which have been found in all PP5s by this time existed in the N-terminal region of the protein. Moreover, the C-terminal region of the protein contains a highly conserved type 2A serine/threonine protein phosphatase domain (PP2A), within which motifs (-GDXHGQ-, -GDXVXRG- and -RGNHE-) necessary for the activity of serine/threonine phosphatase were included (see FIG. 3). The three conserved motifs play important roles in catalysis, substrate binding and metal ion binding (Ollendorff, V. *et al.*, *J. Biol. Chem.*, 272:32011–32018, 1998). In

addition, the C-terminal region of the protein included consensus sequence "SAPNYC" (Ollendorff, V. *et al.*, *J. Biol. Chem.*, 272:32011-32018, 1998) that binds to an okadaic acid, thereby inhibiting enzyme activity (see FIG. 3). From the above results, it could be found that the cDNA clone encodes a serine/threonine protein phosphatase.

The present inventors named the cDNA clone "*PAPP5*" (phytochrome-associated protein phosphatase 5). The full-length cDNA sequence of *PAPP5* and an amino acid sequence deduced therefrom are set forth in SEQ ID NO: 3 and SEQ ID NO: 4, respectively.

Meanwhile, the results of Northern blot analysis revealed that cDNA of *PAPP5* is similar to a single transcript having a total length of about 2kb, and the result of Southern blot analysis confirmed that *PAPP5* is a single copy gene (data not shown).

<Example 3>

Examination of molecular specificity of interaction between PAPP5 and phytochromes

A pGilda vector and a pJG4-5 vector (OriGene Technologies) were used to prepare a prey and a bait, respectively (see FIG. 4). In this case, as control groups to examine specific binding, hepatitis C virus (HCV) protein NS5A(N) (distributed from professor SK Jang, Department of Life Science, Pohang University of Science and Technology, Korea) and another plant protein SPINDLY(SPY) having the TPR domain (Jacobsen, S. E., *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 93:9292-9296, 1996) were used. Furthermore, the prey and the bait were interchanged to observe protein-protein binding by a reciprocal method. The prepared prey and bait together with a

pSH18-34 reporter plasmid (OriGene Technologies) were co-introduced into an EGY48 yeast strain (OriGene Technologies). Next, the transformants were selected on a media containing X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; Rose Scientific Ltd.). Plate-growth assays were performed on Leu-drop out media and X-gal media.

As a result, it could be seen that PAPP5 did not interact with the NS5A(N) protein of HCV whereas it interacted specifically with *Arabidopsis thaliana* phytochrome A (see FIG. 4). In addition, another plant protein SPY having the TPR domain did not interact with the phytochrome. This suggests that the binding between PAPP5 and phytochromes is a very specific binding.

<Example 4>

***In vitro* binding assay**

<4-1> Expression and purification of GST-PAPP5 fusion protein

In order to examine the *in vitro* interaction between PAPP5 and phytochromes, a vector for expressing a full-length *PAPP5* was constructed using a pGEX4T-1 vector (Amersham Pharmacia Biotech.). First, the full-length *PAPP5* was amplified by PCR using primers set forth in SEQ ID NO: 5 and DEQ ID NO: 6. The PCR reaction consisted of predenaturation of template DNA for 5 min at 94 °C, and then, 30 cycles of 30 sec at 94 °C, 30 sec at 50 °C and 1 min at 72 °C, followed by 10 min at 72 °C. The PCR product was cloned into a pGEX4T-1 vector (Amersham Pharmacia Biotech.). The recombinant vector containing *PAPP5* was introduced into *E. coli* BL21. The transformants were cultured with 1 mM IPTG to express a GST-PAPP5 fusion protein. The expressed GST-PAPP5 fusion protein was purified using glutathione-sepharose 4B beads (Amersham Pharmacia Biotech)

(Skinner, J. *et al.*, *J. Biol. Chem.*, 272:22464-22471, 1997). In order to purify the protein to a native state, the GST-PAPP5 fusion protein was eluted with a buffer solution containing 50 mM Tris-HCl (pH8.0), 4 mM $MnCl_2$, 0.1% β-mercaptoethanol and 10 mM glutathione. Then, it was dialyzed with a solution containing 25% glycerol, 1 mM EGTA, 0.1% β-mercaptoethanol, 20 mM Tris-HCl (pH7.6) and 4 mM $MgCl_2$, at 4 °C overnight. The dialyzed protein sample was stored at –20 °C until use for analysis.

<4-2> Construction of PHYA and PHYB expression vectors

An *in vitro* transcription/translation system was used to express apoproteins PHYA and PHYB of *Arabidopsis thaliana* phytochromes A and B.

First, in order to synthesize PHYA *in vitro*, the full-length cDNA of PHYA containing *Bam*HI and *Xho*I restriction enzyme recognition sequences at both the ends was amplified by PCR using primers set forth in SEQ ID NO: 7 and SEQ ID NO: 2. The PCR reaction consisted of predenaturation of template DNA for 5 min at 94 °C, and then, 30 cycles of 30 sec at 94 °C, 30 sec at 50 °C and 2 min and 30 sec at 72 °C, followed by 10 min at 72 °C. Thereafter, the PCR product was inserted into the *Bam*HI-*Xho*I site of a pTriEx-1 vector (Novagen), thus preparing a recombinant expression vector for the expression of PHYA. Meanwhile, for the synthesis of PHYB, a PCR product having the sequence of *Fba*I-full length *PHYB* cDNA-*Eco*52I was amplified using primers set forth in SEQ ID NO: 8 and SEQ ID NO: 9. This PCR product was inserted into the *Bam*HI-*Xho*I site of a pTriEx-1 vector, thus preparing a recombinant expression vector for the expression of PHYB. Each of the encoded proteins was synthesized *in vitro* using ^{35}S-labeled methionine. The

synthesis was performed using a reticulocyte TnT transcription/translation system (Promega) according to the manufacturer's recommendation.

<4-3> *In vitro* binding assay

1 μg of the GST-PAPP5 fusion protein prepared in Example <4-1> and 10 μl of each of the TnT proteins prepared in Example <4-2> together with a protease inhibitor (Complete, Roche Diagnostics GmbH) were added to a 0.3 ml of binding buffer solution (20 mM Tris-HCl, pH7.5, 150 mN NaCl, 1 mM dithiothreitol, 0.1% Tween 20). Then, the mixture was shaked weakly at 4 °C to induce a binding reaction. After adding 10 μl of glutathione-sepharose 4B beads (Amersham Pharmacia Biotech. AB) to the mixture, the reaction was further performed for one hour in the same condition. After centrifugation, the supernatant fraction was separately stored, and the pellet (sepharose bead fraction) was washed three times with 1 ml of binding buffer solution. The pellet and supernatant fractions were analyzed on 10% acrylamide-containing SDS-PAGE gel. Next, visualization was performed using Fuji FLA-2000R image analyzer (Fuji Photo Film).

As a result, it could be seen that PAPP5 has the property of binding to the phytochromes A and B of *Arabidopsis thaliana* (see FIG. 5).

<Example 5>

Examination of interaction between TPR domain of PAPP5 and phytochromes

PP5s comprising the inventive PAPP5 protein are distinguished from other members of the PP1/PP2 group in that they contain a unique N-terminal domain consisting of several TPRs. TPR motifs are assumed to form amphipathic helices

and known to mediate protein-protein interaction (Das, A. K., *et al.*, *EMBO J.*, 17:1192-1199, 1998; Skinner, J. *et al.*, *J. Biol. Chem.*, 272:22464-22471, 1997; Ollendorff, V. *et al.*, *J. Biol. Chem.*, 272:32011-32018, 1998; Chinkers, M. *Trends Endocrinol. Metab.*, 12:28-32, 2001). Thus, in order to examine whether TPRs are involved in the interaction between the inventive PAPP5 protein and the phytochrome molecules, a pull-down assay and a quantitative yeast 2-hybrid interaction assay were performed.

<5-1> Pull-down assay

According to the same method as in Example <4-1>, each of recombinant expression vectors for the TPR domain of PAPP5 consisting of the sequence of amino acids 1-138 of SEQ ID NO: 4 (N-terminal domain; GST-TPR) and for the PP2A enzymatic domain of PAPP5 with a deletion of amino acids 1-138 in SEQ ID NO: 4 (C-terminal domain; GST-PP2Ac) was constructed using the pGEX4T-1 vector (Amersham Pharmacia Biotech). The TPR domain of PAPP5 was amplified by PCR using primers set forth in SEQ ID NO: 5 and SEQ ID NO: 10. The PCR reaction consisted of predenaturation of template DNA for 5 min at 94 °C, and then, 30 cycles of 30 sec at 94 °C, 30 sec at 50 °C and 1 min at 72 °C, followed by 10 min at 72 °C. Meanwhile, the PP2A catalytic domain of PAPP5 was amplified by PCR using primers set forth in SEQ ID NO: 6 and SEQ ID NO: 11. This PCR reaction consisted of predenaturation of template DNA for 5 min at 94 °C, and then, 30 cycles of 30 sec at 94 °C, 30 sec at 50 °C and 1 min at 72 °C, followed by 10 min at 72 °C. Thereafter, according to the same method as in Example <4-1>, an *in vitro* assay was performed on each of the proteins which have been expressed and purified in *E. col.*

As a result, as shown in FIG. 6A, it was found that only the TPR domain of PAPP5 fused with GST (GST-TPR) has the ability to interact with the phytochrome molecules. This indicates that the TPR domain is involved in the interaction between PAPP5 and phytochrome molecules.

<5-2> Quantitative yeast two-hybrid assay

In order to quantitatively assay the interaction between the TPR domain of PAPP5 and phytochromes, each of recombinant vectors (preys) for the TPR domain of PAPP5 and for the PP2A catalytic domain of PAPP5 were prepared, respectively.

A *PHYA* gene or a *PHYB* gene was prepared by performing PCR according to the same method as in Example <4-2>, and inserted into a pGilda vector. The recombinant vector was used as a bait. The prepared bait or prey was co-introduced into an EGY48 yeast strain together with a pSH18-34 reporter plasmid. The transformed strain was cultured until the OD_{600} value reached about 0.7. The cultured cells were washed one time with Z-buffer solution (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, pH 7.0), and then re-suspended in the solution. Thereafter, the solution was frozen with liquid N_2. The frozen solution was dissolved, to which β-mercaptoethanol and ONPG (o-nitrophenyl-β-D-galactopyranoside) were then added at final concentrations of 0.2% and 0.67 mg/ml, respectively. The dissolved solution was allowed to react for 30 minutes at 37 °C. Next, Na_2CO_3 was added to the solution at a final concentration of 0.3 M to terminate the reaction. The absorbance at 420 nm was measured, and the β-galactosidase activity was determined in Miller units (Miller, J. H. *Experiments in Molecular Genetics* (Cold Spring Harbor Laboratory, Cold Spring harbor, New York, 1972)).

As a result, as shown in FIG. 6B, the TPR domain-deleted PP2A catalytic domain of PAPP5 (B42-PP2Ac) showed a remarkable reduction in the activity of interaction with phytochromes. This result reconfirms that the TPR domain of PAPP5 is a specific region for the interaction between PAPP5 and phytochrome molecules. Also, this coincides with the result of the pull-down assay.

<Example 6>

***In vitro* phosphatase assay**

<6-1> Measurement of phosphatase activity

In order to examine whether the inventive PAPP5 protein has enzymatic activity, an enzymatic assay was performed using inorganic ρ-nitrophenyl phosphate (ρNPP) as a substrate. First, the GST-PAPP5 fusion protein purified in Example <4-1> was prewarmed at 30 °C for 1 minute. The prewarmed enzyme solution was added to 100 μl of kinase/phosphatase (KP) buffer (20mM Tris-HCl pH 7.5, 30 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 0.1% β-mercaptoethanol, 0.1% ethanol) containing O-400 mM ρNPP to initiate a reaction. To examine the autohydrolysis of ρNPP at each substrate concentration, the GST-PAPP5 enzyme solution was not added to a control group. After reaction at 30 °C for 15 minutes, 900 μl of 0.25N NaOH was added to terminate the reaction. The absorbance at 410 nm was measured. After subtracting the absorbance value of the control reaction solution containing all the components except for the enzyme from that of the reaction solution containing the enzyme, the rate was calculated using a millimolar extinction coefficient (17.8) of ρ-nitrophenolate ions.

As a result, as shown in FIG. 7A, it could be seen that the phosphatase activity was increased with an increase in the concentration of ρNPP. This indicates

that the inventive PAPP5 protein has phosphatase activity. A an enzyme-substrate reaction in the presence of 100 μM arachidonic acid was calculated as a Km and Vmax of Michaelis-Menten kinetic, and as a result, Km was 160 mM ρNPP and Vmax was 22μmol Pi released/min/mg.

<6-2> Measurement of induction of catalytic activity of PAPP5 by arachidonic acid

The most distinguishable characteristic of PP5s is that their catalytic activity is induced by arachidonic acid (Das, A. K., *et al.*, *EMBO J.*, 17:1192-1199, 1998; Skinner, J. *et al.*, *J. Biol. Chem.*, 272:22464-22471, 1997; Ollendorff, V. *et al.*, *J. Biol. Chem.*, 272:32011-32018, 1998; Chinkers, M. *Trends Endocrinol. Metab.*, 12:28-32, 2001). In order to confirm if the phosphtase activity of the inventive PAPP5 protein is activated by arachidonic acid, 0-300 μM arachidonic acid was added to a reaction mixture in the presence of 100mM ρNPP, and the enzymatic activity was measured according to the same method as Example <6-1>.

As a result, the activity of GST-PAPP5 was increased in a concentration-dependent manner by arachidonic acid, and reached the stationary phase at concentration above about 100 μM of an arachidonic acid (see FIG. 7B).

Furthermore, the catalytic activity of PP5s is known to be inhibited by okadaic acid *in vitro* (Das, A. K., *et al.*, *EMBO J.*, 17:1192-1199, 1998; Skinner, J. *et al.*, *J. Biol. Chem.*, 272:22464-22471, 1997; Ollendorff, V. *et al.*, *J. Biol. Chem.*, 272:32011-32018, 1998; Chinkers, M. *Trends Endocrinol. Metab.*, 12:28-32, 2001). Thus, the inhibitory effect of okadaic acid on the phosphatase activity of GST-PAPP5 was examined. As a result, the IC_{50} of okadaic acid, at which the enzyme activity is inhibited by 50%, was 5 nM (data not shown).

These results demonstrate that the inventive PAPP5 encodes a protein belonging to the PP5 subfamily of the serine/threoine protein phosphatase family.

<6-3> Examination of enzymatic activity by allosteric conformational structure change

The results of previous studies on PP5s isolated from other species revealed that the N-terminal fragment of PP5s, which contains TPR motifs, shows an allosteric conformational change (Das, A. K., *et al.*, *EMBO J.*, 17:1192-1199, 1998; Skinner, J. *et al.*, *J. Biol. Chem.*, 272:22464-22471, 1997; Ollendorff, V. *et al.*, *J. Biol. Chem.*, 272:32011-32018, 1998; Chinkers, M. *Trends Endocrinol. Metab.*, 12:28-32, 2001). Thus, in order to confirm if PAPP5 also have this property, the present inventors examined the phosphatase activities of GST-PAPP5, GST-TPR and GST-PP2Ac in the same manner as in Example <6-1>. At this time, the concentration of pNPP was 100 mM.

As a result, as shown in FIG. 7c, it could be seen that the phosphatase activity of GST-PAPP5 was about five times increased by arachidonic acid. On the other hand, GST-PP2Ac with a deletion of the TPR domain (amino acids 1-138) showed five times higher activity than that of GST-PAPP5 with no arachidonic acid, regardless of the addition of arachidonic acid.

These results indicate that the phosphatase activity of PAPP5 undergoes regulation by the allosteric conformational change of the TPR domain, similarly to other PP5s, while the TPR domain also has an autoinhibitory activity. Also, these results suggest that the TPR domain-deleted PP2A catalytic domain of PAPP5 can be used as a phosphatase.

<Example 7>

Examination of plant phenotypes by inactivation or overexpression of PAPP5

In order to examine whether PAPP5 is involved directly in phytochrome-mediated light signal transduction *in vivo*, a knock-out mutant of a *PAPP5* gene and a PAPP5-overexporessing plant were produced and examined for their phenotypes.

<7-1> Screening of knock-out mutants of *PAPP5* gene

Two mutants carrying a T-DNA insertion within the *PAPP5* gene were identified to establish two mutant lines (*papp5-1* and *papp5-2*).

One mutant *papp5-1* was obtained from a separate T-DNA mutagenized population (SIGnAL T-DNA Express (http://signal.salk.edu/cgi-bin/tdnaexpress), Salk Institute Genomic Analysis Laboratory) prepared from Col-0 wild-type plants. The T-DNA in the mutant was found to be inserted into 1st intron (see FIG. 8A), and from this fact, *papp5-1* was assumed as a null allele. Another mutant *papp5-2* was isolated by screening DNAs isolated from a knock-out mutant population (Krysan, P. J., *et al.*, *Plant Cell,* 11:2283-2290, 1999) prepared from Ws-2 wild-type plants. The T-DNA in *papp5-2* was found to be inserted into 12nd intron (see FIG. 8A).

Segregation examination using drug-resistant markers (kanamycin-resistent genes) was conducted, and the results showed that both *papp5-1* and *papp5-2* had single T-DNA inserted into the *PAPP5* locus. Several drug-resistant seedlings for the two mutant lines having the null allele were propagated. Then, homozygous lines were identified by screening for the drug resistance in the progeny of individual plant. The homozygosities of the mutant lines were confirmed either by Southern blot analysis or PCR (data not shown).

<7-2> Construction of PAPP5-overexpressing plants

PAPP5 cDNA was amplified by PCR using primers set forth in SEQ ID NO: 12 and SEQ ID NO: 13. The PCR reaction consisted of predenaturation of template DNA for 5 min at 94 °C, and then, 30 cycles of 30 sec at 94 °C, 30 sec at 50 °C and 1 min at 72 °C, followed by 10 min at 72 °C. Thereafter, the PCR product was cloned into a pNB96 vector having a dual 35S CaMV promoter and followed by a 35S CaMV terminator (35S ter). The recombinant vector was introduced into *Agrobacterium* strain AGL1 by electroporation. Next, according to the floral-dip method (Clough, S. J. & Bent, A. F. *Plant J.*, 16:735-743, 1998), *Arabidopsis thaliana* was transformed with the transformed *Agrobacterium*. 25 μg/ml of DL-PPT (DL-phosphinothricin, Duchefa Biochemie BV) was used to select two independent transgenic plants overexpressing PAPP5. The two selected plants were named "*PAPP5-OX1*" and "*PAPP5-OX2*", respectively.

<7-3> Confirmation and analysis of knock-out mutants and overexpression plants

a) Northern blot analysis

For analysis of *PAPP5* transcript levels, total RNA from the plants obtained in Examples <7-1> and <7-2> was isolated using RNeasy plant mini kits (Qiagen, Valencia, CA). 10 μg of the total RNA was separated on 1.0% agarose gel containing formaldehyde, and subsequently transferred to a nylon membrane. The membrane was hybridized with a ^{32}P-labeled probe specific to *PAPP5* gene (the 300-700 bp fragment of SEQ ID NO: 3). The radioactive signals were quantified by Fuji FLA-2000R image analyzer (Fuji photo film).

As a result, as shown in FIG. 8B, the radioactive signals were not detected in the knock-out mutants *papp5-1* and *papp5-2* whereas it was detected in the overexpression plants, *PAPP5-OX1* and *PAPP5-OX2*.

b) Examination of photoresponsiveness

Seeds of the knock-out mutants and overexpression plants were surface-sterilized with 30% bleach (1.2% sodium hypochlorite) and 0.015% Triton X-100 for 10 minutes. Then, the seeds were washed five times with sterile water. For vernalization, the seeds were exposed to dark/cold-treatment for 3 days, and then placed on 0.8% phytoagar containing growth medium (0.1X Murashige-Skoog without sucrose; 0.1X MS). Then, the plate was exposed to continuous white light with an intensity of 200 $\mu molm^{-2}sec^{-1}$ (F48T12/CW/VHO, Philips) for 12 hours, and then incubated in dark conditions at 22 °C for 12 hours to promote germination. Before measuring the length of hypocotyl, the plate was placed under the various conditions (dark conditions, continuous red light (Rc) irradiation and continuous far-red-light (FRc) irradiation) for 4 days. Here, the light source was used as described in Kim, B. C. *et al. Plant J.*, 9:441-456, 1996, and the fluence rate was monitored using a spectroradiometer (Hanbead Optical Power Meter 840, Newport). The length of hypocotyl was measured using a HP ScanJet 5370C digital scanner (Hwelett Packard) and Scion image software (Beta 4.0.2, Scion Corporation). Also, wild-type *Arabidopsis thaliana* (Col-0) and phytochrome mutants (*phyB-9* and *phyA-211*) (distributed from *Arabidopsis* Biological Resource Center) were used as control plants.

FIG. 9 shows the curves of inhibition of hypocotyl length in the knock-out mutant (*papp5-1*) and the PAPP5-overexpressing plant (*PAPP5 OX-2*) according to Rc and FRc fluence rates, compared to those in the wild-type plant (Col-0) and the phytochrome mutant (*phyB-9*), in order to examine the photoresponsiveness of the plants. As shown in FIG. 9, the knock-out mutant *papp5-1* showed the hyposensitivity to Rc and FRc of at lower fluence rate, whereas the PAPP5-overexpressing plants showed the hypersensitivity to Rc and FRc. Also, in the PAPP5-overexpressing plants, an "End-Of-Day Far-Red" (EOD-FR)" response which is a phytochrome B-mediated response, and anthocyanin accumulation which is a phytochrome A-mediated response, were stronger than those in the wild-type plant (data not shown). It was found that the intensities of such light-dependent phenotypes were correlated with the *PAPP5* transcript level.

Meanwhile, the knock-out mutant induced by the disruption of the *PAPP5* gene showed long hypocotyls phenotype, diminished rates of light-induced hook opening and cotyledon separation, reduced cotyledon extension, and early flowering (data not shown). This suggests that PAPP5 is functionally involved in the photomorphogenesis of plants, which is regulated by phytochromes.

<Example 8>

Analysis of transcript levels of photoresponsive genes in knock-out mutants and overexpression plants

To determine whether the involvement of PAPP5 in phytochrome-mediated light signal transduction be observed at a gene expression level, the expression levels of the following three genes whose expressions have been known to be regulated by light were examined in the knock-out mutants and the overexpression plants,

compared to those in the wild-type plant: positively regulated genes, *RBCS* (GenBank accession No. X15221), *CAB2* (GenBank accession No. X14564), and *CHS* (GenBank accession No. BT000596).

The seeds of each of the plants were placed individually onto 0.8% phytoagar plate containing 0.1X MS salts, and the plate was kept at 4 °C in dark condition for 3 days. In order to induce germination, the plate was exposed to white light with an intensity of 200 $\mu molm^{-2}sec^{-1}$ for 24 hours. Next, the seedlings were grown in dark conditions for further 4 days, and then, subsequently transferred to the following light conditions:

Rc: exposure at a wavelength of 664 nm and a quantity of light of 20 $\mu molm^{-2}sec^{-1}$ for 2 hours; and

FRc: exposure at a wavelength of 748 nm and a quantity of light of 10 $\mu molm^{-2}sec^{-1}$ for 2 hours.

After tissue was harvested in green safe light, total RNA was isolated using the RNeasy plant mini kits (Qiagen) from the tissue. Then, 5 μg of the total RNA was separated on 1% agarose gel containing formaldehyde, and the gel was transferred to a nylon membrane. The membrane was hybridized with ^{32}P-labled probes specific to the each gene. Signals were quantified by a Fuji FLA-2000R image analyzer (Fuji Photo Film).

As a result, as shown in FIG. 10, upon dark treatment, *RBCS, CAB2* and *CHS* mRNA were detected at low levels in all the plants. However, in the wild-type plants (Col-0, and Ws-2), the expression levels of the three genes were increased to high levels upon Rc and FRc irradiation. This coincides with the fact that phytochrome A and phytochrome B are involved in the regulation of these genes (Nagy, F. & Schafer, E. *Annu. Rev. Plant Biol.*, 53:329-355, 2002). Meanwhile,

upon Rc and FRc irradiation, the PAPP5-overexpressing plant showed an increase in the expression levels of the genes as compared to the wild-type plant. On the other hand, in the knock-out mutants (*papp5-1,* and *papp5-2*), the expression levels of the genes were increased as compared to the case of dark treatment, but lower than that of the wild-type plant. This indicates those the photosensitivities of the *RBCS, CAB2* and *CHS* genes in the knock-out mutants are lower than those in the wild-type plant. From the above results, it could be found that, due to the *PAPP5* null mutation, the ability of phytochromes to transduce signals to the photosensitive genes was reduced.

<Example 9>

Dephosphorylation of autophosphorylated phytochrome by PAPP5

It was previously shown, using biochemical analyses, that reversible phosphorylation/de phosphorylation may be involved in the signal transduction and/or regulation of phytochrome activity (Yeh, K. C. & Lagarias, J. C. *Proc. Natl. Acad. Sci. U.S.A.* 95:13976-13981, 1998; Fankhauser, C. *et al., Science,* 284:1539-1541, 1999). Thus, in order to examine the molecular nature of PAPP5 interacting with phytochromes, the present inventors tested whether autophosphorylated phytochrome A can be directly dephosphorylated by PAPP5 *in vitro*.

First, KP buffer (20 mM Tris-HCl pH7.5, 30 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 0.1% β-mercaptoethanol, 0.1% ethanol) containing 1 μg of purified oat phytochrome A (provided from Kumho Life and Environmental Science Laboratory, Korea) was set on ice and irradiated with Rc light output of 50 $\mu molS^{-1}m^{-2}$ at 664 nm for 5 minutes. Then, the reaction solution was allowed to react with [γ-^{32}P]ATP in dark conditions for 30 minutes (autophosphorylation of phytochrome A). Following addition of 1 μg of each of the fusion proteins GST-PAPP5, GST-TPR and GST-

PP2Ac prepared in Examples <4-1> and <5-1>, the reaction continued for more 30 minutes, and then stopped by adding 12 μl of 5X Tris-glycine SDS sample buffer under dim green safe light. The resulting reaction product was subjected to 10% SDS-PAGE, followed by autoradiography analysis with Fuji FLA-2000R image analyzer (Fuji Photo Film).

As a result, as shown in FIG. 11A, only GST-PAPP5 dephosphorylated the autophosphorylated oat Pfr phytochrome A by about 80%. Although the dephosphorylation of oat phytochrome A was also detectable in the reaction mixture containing the GST-PP2Ac, the level of dephosphorylation was very low. This suggests that PAPP5 dephosphorylates the autophosphylated Pfr-phytochrome as a main target, and thereby modulating the phosphorylation status of the Pfr-phytochrome. Namely, these results indicate that the autophosphorylated Pfr phytochrome A induces the phosphatase activity of PAPP5, and the activated PAPP5 directly dephosphorylates the autophosphorylated Pfr-phytochrome A. Also, these results indicate that simple physical association of TPR domain (GST-TPR) of PAPP5 or non-interactive catalytic domain of PAPP5 (GST-PP2Ac) do not affect the phosphorylation status of the Pfr-phytochrome.

Next, the present inventors investigated whether the phosphatase activity of PAPP5 depends on a change in the spectroscopic structure (photoconversion) of phytochrome molecules (Pr↔Pfr). KP buffer solution containing phytochrome was set on ice and irradiated with a Rc light output of 50 $\mu molS^{-1}m^{-2}$ at 664 nm (conversion of phytochrome into Pfr form) or a FRc light output of 50 $\mu molS^{-1}m^{-2}$ at 748 nm (conversion of phytochrome into Pfr form) for 5 minutes. Then, the autophosphylation/dephosphorylation test of phytochrome was performed in the same manner as described above.

As a result, as shown in FIG. 11B, the dephosphorylation by PAPP5 was higher in Pfr phytochrome than that in Pf phytochrome.

The above results suggest that the phosphatase activity of PAPP5 for phytochrome is photoregulated, occurring predominantly in the phosphorylated Pfr-form. This correlation between the formation of the phytochrome-PAPP5 complex and the activation/stimulation of phosphatase activity suggests that PAPP5 specifically targets the phosphorylated Pfr-form of phytochrome by feedback manner.

<Example 11>

Production of plants overexpressing TPR domain of PAPP5

A reverse genetic approach for dominant negative mutation was used to investigate the roles of TPR domain of PAPP5. The TPR domain-coding region (the sequence of amino acids 1-138 of SEQ ID NO: 4) was amplified by PCR using primers set forth in SEQ ID NO: 5 and SEQ ID NO: 10. The PCR reaction consisted of predenaturation of template DNA for 5 min at 94 °C, and then, 30 cycles of 30 sec at 94 °C, 30 sec at 50 °C and 1 min at 72 °C, followed by 10 min at 72 °C. Thereafter, the PCR product was cloned into a pNB96 vector (see FIG. 12A). The prepared vector was named "pNB96-TPR". The pNB96-TPR vector was introduced into *Agrobacterium* strain AGL1 by electroporation. Next, according to the floral-dip method (SIGnAL T-DNA Express (http://signal.salk.edu/cgi-bin/tdnaexpress), Salk Institute Genomic Analysis Laboratory), *Arabidopsis thaliana* was transformed with the transformed *Agrobacterium*. 25 μg/μl of DL-PPT (Duchefa Biochemie BV) was used to select transgenic plants. Total 25 T1 lines were obtained and named "PAPP5-DN". Thereafter, on the basis of segregation of DL-PPT resistance,

homozygous T3 seeds were isolated. The T3 plants were grown in the white light condition, and the resulting plants have shorter height, multiple shoots, and dwarfing of the floral shoot internodes, as compared to wild-type plants (see FIG. 12B). This is thought to be induced by improved protein-protein interaction, in view of the fact that the TPR domain of PAPP5 is involved in protein-protein interaction. Furthermore, this indicates that the introduction and overexpresison of the TPR domain of PAPP5 in plants can introduce dwarf characteristics into the plants.

Industrial Applicability

As described above, it was found a novel protein involved in light signal transduction regulating the growth and development of plants and its functions in the present invention. The inventive PAPP5 protein interconnects with phytochromes A and B. The PAPP5 protein contains a TPR domain involved in interaction with phytochromes, and a PP2A catalytic domain having phosphatase activity. The PAPP5 protein can be used as phosphatase. It is also useful in the production of plants sensitive to light signal transduction. Furthermore, the TPR domain present in the PAPP5 protein is useful in the production of dwarf plants.